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Purification of DNA from the cell-associated herpesvirus Marek's disease virus for 454 pyrosequencing using micrococcal nuclease digestion and polyethylene glycol precipitation

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A B S T R A C T

Methods for the isolation of DNA from cell-associated herpesviruses have often yielded samples contaminated with host cellular DNA. Because 2nd and 3rd generation nucleotide sequencers do not rely on molecular cloning of viral DNA, there is a need to develop methods for isolating highly pure DNA from these viruses. The cell-associated alphaherpesvirus Marek's disease virus (MDV-1) was chosen as a test virus for the development of such methodologies. The genomes of six MDV-1 strains have previously been sequenced using both Sanger dideoxy sequencing and 454 Life Sciences pyrosequencing. These genomes largely represent cell culture adapted strains due to the difficulty in obtaining large quantities of DNA from true low passage isolates. There are clear advantages in analyzing MDV-1 virus taken directly from infected tissues or low passage isolates since serial passage attenuates the virus. Procedures using an ATP-dependent exonuclease and Phi29 DNA polymerase to degrade host DNA selectively and amplify MDV-1 DNA enzymatically from total DNA preps were attempted without much success. Ultimately, however, a protocol was developed for purification of low passage MDV-1 DNA from infected avian fibroblasts. The method builds upon and extends available protocols based on hypotonic lysis to release virus particles followed by micrococcal nuclease treatment to degrade cellular DNA. Intact high-molecular weight viral DNA is purified away from an excess of degraded cellular DNA using polyethylene glycol precipitation. 454-based pyrosequencing of viral DNA purified in this manner has generated data containing as little as 2.3% host sequence. On average, DNA preparations were 70% (+/−20%) pure yielding a genome coverage range of 25–74-fold.

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1. Introduction

Marek's disease is an immunosuppressive and neoplastic disease characterized by the development of T-cell lymphomas in susceptible chickens and is caused by gallid herpesvirus type 2 (GaHV-2), commonly referred to as Marek's disease virus (MDV-1) (Osterrieder et al., 2006). Much progress has been made in the characterization of the virus, its genome and gene products despite difficulties in isolating DNA from this avidly cell-associated herpesvirus. Methods for the purification of DNA from cells infected with herpesviruses that produce high titers (10^9 pfu/ml) of free virus (e.g. herpes simplex virus-1) have been described and adapted for use with cell-associated viruses with limited success (Gilden et al., 1982). Several variations of the "Hirt Supernatant" method, originally described in 1967 for the isolation of polyoma DNA (Hirt, 1967), have been reported for the isolation of herpesviral DNA (Eizuru et al., 1984; Pater et al., 1976; Pignatti et al., 1979; Rosenthal et al., 1983; Walboomers and Schegget, 1976). These methods are based on the preferential precipitation of denatured cellular DNA/protein complexes and subsequent purification of the viral DNA remaining in the supernatant. In other methods nucleocapsids and virions are physically or chemically released from cells and viral particles are subsequently separated from cellular debris by differential centrifugation (Esposito et al., 1981; Hayward et al., 1975; Wechsler et al., 1985). However, these preparations can still be contaminated with cellular DNA due to co-pelleting of the DNA along with the viral particle. Removal of cellular DNA using density gradient separation has not been possible due to the similar densities of chicken and GaHV-2 DNA (1.702 and 1.705 g cm^{-3}, respectively). To remove contaminating cellular DNA, protocols have evolved to include a nuclease step either before centrifugation or after (Greenberg and Bender, 1997; Muggeridge and Fraser, 1986; Sinzger et al., 1999; Spiker et al., 1983; Su et al., 2002).

To aid in comparative genomic efforts to understand the genetic basis of GaHV-2 virulence, various methods were examined for

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the isolation and enzymatic amplification of viral DNA suitable for 454 pyrosequencing, a technology that does not rely on shotgun cloning of fragmented DNA. The ultimate goal was to develop a technique that would yield pristine GaHV-2 DNA with limited cellular DNA contamination and which does not rely on extensive serial propagation in cell culture, a process which has been shown to attenuate the virus. Three approaches were examined: (1) Phi29 DNA polymerase-directed amplification of GaHV-2 DNA using a genome-spanning collection of GaHV-2-specific oligonucleotides and whole infected cell DNA preps; (2) ATP-dependent exonuclease degradation of non-covalently closed circular DNA (cellular and linear GaHV-2) prior to Phi29 DNA polymerase-directed amplification; and (3) hypotonic lysis of infected cells, micrococcal exonuclease degradation of non-encapsulated DNA and removal of degraded DNA using polyethylene glycol precipitation.

2. Materials and methods

2.1. Viruses and cells

Aliquots of GaHV-2 strain 648A representing passages 10, 30, 40, 60, 80 and 100 were prepared as described previously (Gimeno et al., 2001) and were kindly provided by B. Riegle (USDA-ARS-ADOL, E. Lansing, MI). Strain CU-2 was obtained from Karl A. Schat (Cornell University, Ithaca, NY). A single passage of 648A in secondary avian fibroblasts to yield p11, p31, p41, p61, p81 and p101 was done mainly in chick embryo fibroblasts (CEFs). However, primary duck embryo fibroblast cells were also used to propagate p10 due to low DNA yields when this passage was propagated on CEFs.

Secondary CEFs (10^7) were seeded overnight in a 75-cm^2 flask in DMEM (Gibco) supplemented with 8% fetal bovine serum (FBS), glutamine (292 ug/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) under 5% CO_2 at 39 °C. CEFs were infected at a multiplicity of infection (m.o.i.) of 1.0 with CU-2 or passages of 648A in complete medium containing 2% FBS. Eighteen hours postinfection the serum concentration was reduced to 0.2%. For propagation of p10, secondary DEFs were seeded in complete medium containing 4% newborn calf serum (NCS) and infected in complete medium containing 2% NCS. CEFs and DEFs were harvested when cytopathic effect was >80%, usually 4–6 days postinfection.

2.2. Extraction of GaHV-2 DNA for Phi29 DNA polymerase amplification

Total cellular DNA was extracted from CEFs infected with strain CU-2 using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, 200 μl of infected cellular suspension was treated with proteolytic enzymes at 56 °C for 10 min. An equal volume of 100% ethanol was added to the lysate and the DNA was purified by affinity chromatography. Contaminating proteins were removed via ethanol/salt washes and the DNA was eluted in 10 mM Tris, pH 8.0 and quantified at 260 nm.

2.3. Phi29 DNA polymerase amplification of whole infected cell DNA preps

Ten nanograms of DNA isolated from GaHV-2 infected cells were denatured with 1.6 μM of MDV-specific primers (listed in Table 1) in 10 mM Tris–HCl, 0.5 mM EDTA pH 9.0 at 95 °C for 3 min and allowed to cool slowly to room temperature. Bovine serum albumin was added to a final concentration of 200 μg/ml along with 1× Phi29 buffer [50 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 4 mM dithiothreitol, pH 7.5], 4 mM dNTP and 5 U of Phi29 polymerase (NEB, Beverly, MA). The DNA was incubated for 48 h at 30 °C and the reaction was terminated at 65 °C for 10 min. Alternatively, 6.7 μM of random hexamers (Fidelity Systems, Gaithersburg, MD) were used to prime the DNA in ATP-dependent exonuclease-treated preps.

Table 1
Oligonucleotides used for amplification of GaHV-2 DNA.

Name	Sequence	Percent G/C	Tm values (°C)[a]
MDV_phi_01	ATCATACGCGTCAGAFEC	50.0	64.8
MDV_phi_02	TCGGTTATGTCGCAFOG	52.9	65.0
MDV_phi_03	GCATTACGATCGACTEZG	50.0	63.0
MDV_phi_04	TTGCTCGCATAACCTEZC	50.0	65.1
MDV_phi_05	GCTTATCGTCGAAGTEZC	50.0	62.6
MDV_phi_06	TAGTTTCCGTCCGGFZG	52.9	63.5
MDV_phi_07	GCCTTAGATCGTCAAZEC	50.0	63.1
MDV_phi_08	CATAGATCCGCGAAFOG	52.9	62.7
MDV_phi_09	TTCGAACGTCGACCFZC	52.9	64.3
MDV_phi_10	ATGTCCCGAAGAAGFOG	52.9	63.8
MDV_phi_11	AACCCGTGACGATAZOC	52.9	63.6
MDV_phi_12	GTGCGGACGAATTAZEC	52.9	63.7
MDV_phi_13	TTTCCGTCTCGAAGTCEFC	52.6	66.2
MDV_phi_14	GTGTAATACGCGAGZOG	52.9	62.2
MDV_phi_15	GCGATGGTAGACGGAFZC	55.6	64.5
MDV_phi_16	TGCTACAACGACGAZOC	52.9	64.1
MDV_phi_17	CATTCGCGAAGCTAEFG	52.9	62.4
MDV_phi_18	TTCAGTCTACGGTCZEG	52.9	62.8

Oligonucleotides used for the Phi29 DNA polymerase amplification of whole GaHV-2-infected cellular DNA. The Tms were calculated by the nearest-neighbor method using the IDT OligoAnalyzer 3.1 program (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Nucleotide modification codes: F, A-Phosphorothioate; O, C-phosphorothioate; E, G-phosphorothioate; Z, T-phosphorothioate.

[a] Amplification reaction conditions: 1.6 μM oligos, 4 mM dNTP, 10 mM Mg^{2+} 10 mM monovalent salts.

2.4. ATP-dependent exonuclease treatment of whole infected cell DNA preps

Two micrograms of DNA were treated with 17.8 U (500 ng) of ATP-dependent exonuclease (Qiagen, Carlsbad, CA) in a buffer containing 3 mM ATP, 10 mM $MgCl_2$ and 50 mM Tris–HCl, pH 8.0 for 1 h at 37 °C. The reaction was stopped by heating to 80 °C for 20 min. The DNA was then purified with three rounds of phenol/chloroform extraction and precipitated with ammonium acetate and ethanol.

2.5. qPCR conditions

Amplification reactions included 5 μl of DNA template (10 ng), 300 nM of Meq-specific primers (5′-GGAGCCGGAGAGGCTTTATG-3′ and 5′-ATCTGGCCCGAATACAAGGAA-3′), 200 nM of probe (5′-[FAM]CGTCTTACCGAGGATCCCGAACAGG [BHQ-1]-3′ and 12.5 μl of Platinum Quantitative PCR System-UDG 2× reaction mix (Invitrogen, Carlsbad, CA) in a total reaction volume of 25 μl as described previously (Islam et al., 2004). The cycling conditions were 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of denaturation and annealing/extension at 94 °C for 15 s and 60 °C for 1 min, respectively. Amplification and data acquisition were carried out using an ABI7500 Real-Time PCR System. Standard curve R^2 value was 0.9997.

2.6. Cloning and DNA sequencing

Three micrograms of ATP-dependent exonuclease-treated/Phi29-amplified DNA in 750 μl of 10 mM Tris–HCl, 1 mM EDTA, 20% glycerol, pH 8.0, were sheared using a nebulizer (Invitrogen, Carlsbad, CA) at 15 psi for 90 s. These conditions produced fragments averaging 1300 bp. The DNA ends were repaired using 2.5 U of Klenow polymerase (NEB, Beverly, MA) in 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9) and 33 μM each dNTP for 15 min at 25 °C. Blunt-ended fragments were dephosphorylated using 8 U of Antarctic phosphatase (NEB,

Beverly, MA) in 1× Antarctic phosphatase buffer (50 mM Bis Tris-Propane, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 6.0) for 15 min at 37 °C. The enzyme was inactivated for 5 min at 65 °C. Dephosphorylated fragments were cloned into the vector pCR-BluntII-TOPO using the conditions defined by the manufacturer (Invitrogen, Carlsbad, CA). Ligated products were transformed into *E. coli* DH5α cells and selected on LB plates containing ampicillin (100 μg/ml) and a topically added cocktail of IPTG (8 μl of 100 mg/ml) and X-gal (40 μl of 20 mg/ml). Clones were screened using a conventional plasmid mini-prep procedure and inserts were sequenced using the BigDye terminator cycle sequencing protocol and analyzed on a model ABI-3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA).

2.7. Hypotonic lysis and micrococcal nuclease treatment

Infected cells were washed with 1× PBS and the rinse pooled with the saved DMEM. Cells were trypsinized with 2.5 ml of Trypsin–EDTA (0.4%) (Gibco) and washed with DMEM containing 8% serum to inactivate the trypsin. Media containing trypsinized cells were again pooled with the saved PBS/MEM mixture. Cells were pelleted at 250 × *g* for 10 min at 4 °C. After decanting the supernatant, the cell pellet was resuspended in 10 ml of cold 1× PBS and again centrifuged under the same conditions. The supernatant was then removed and the pellet was resuspended in 5 ml of cold permeabilization buffer [320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.5) and 1% Triton X-100] and placed on ice for 10 min. Nuclei were pelleted by centrifugation at 1300 × *g* at 4 °C for 15 min. After decanting of the supernatant the pellet was resuspended in 5 ml cold permeabilization buffer and recentrifuged under the previous conditions. After complete removal of the supernatant by aspiration, the nuclei were resuspended in 50 μl of nuclei buffer [10 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$ and 10% sucrose] and mixed with an equal volume of 2× nuclease buffer [40 mM PIPES (pH 7.0), 7% sucrose, 20 mM NaCl, 2 mM $CaCl_2$, 10 mM 2-mercaptoethanol and 200 μM PMSF] containing 150 U of freshly added micrococcal nuclease (USB, Cleveland, Ohio) and 1.0 μl of RNase A (100 mg/ml). Cellular and unpackaged viral nucleic acids were degraded by incubation for 30 min at 37 °C. The reaction was stopped by the addition of 2.4 μl of 0.5 M EDTA. A 400 μl volume of digestion buffer [100 mM NaCl, 10 mM Tris–HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS] containing 2.5 μl of freshly added proteinase K (20 mg/ml) was then added, mixed gently by tapping and viral DNA was released from nucleocapsids by incubation for 18 h at 50 °C. Proteins were removed by three rounds of phenol/chloroform extraction using equal volumes of phenol/chloroform (50%/50%, v/v) with 5 min centrifugations at 8000 × *g*. DNA was precipitated by the addition of ammonium acetate to a final concentration of 2 M along with 2 vol. of 100% ethanol and centrifuged for 40 min at 16,000 × *g*. After decanting the supernatant, the DNA pellet was washed with 300 μl of ice cold 70% ethanol, centrifuged for 10 min at 16,000 × *g* and allowed to air dry. The DNA was dissolved in 100 μl of 10 mM Tris–HCl, pH 7.5 and quantified at 260 nm.

2.8. Polyethylene glycol precipitation of high-molecular weight GaHV-2 DNA

Five hundred nanograms of nucleocapsid purified/micrococcal nuclease-treated DNA were precipitated at room temperature for 20 min with varying concentrations of (w/v) PEG-8000 (6.0–8.0% at 0.5% intervals) and 10 mM $MgCl_2$. After centrifugation at 16,000 × *g* for 40 min, the supernatant was removed and saved (for later comparisons) and the pellet containing the high MW viral DNA was washed with 500 μl 70% ethanol and re-centrifuged at 16,000 × *g* for an additional 20 min. The supernatant containing the low MW DNA was precipitated with 2 M ammonium acetate/2 vol. ethanol, centrifuged at 16,000 × *g* for 40 min, washed with 500 μl of ice-cold 70% ethanol and centrifuged at 16,000 × *g* for an additional 10 min. Pellets containing high and low MW DNA were air-dried and dissolved in 1× TE buffer, pH 7.5.

3. Results and discussion

3.1. Phi29 DNA polymerase amplification of GaHV-2 DNA

The first method investigated for the isolation of GaHV-2 DNA suitable for 454 pyrosequencing involved the enzymatic amplification of viral DNA directly from infected tissues (total DNA) using a high fidelity polymerase (Phi29 DNA polymerase) and a battery of 18 GaHV-2-specific oligonucleotides (Table 1) with binding sites spread throughout the genome. This polymerase was chosen due to its proofreading, high processivity and strand displacement capabilities (Hutchison et al., 2005). It was reasoned that specificity could be engineered into the annealing oligonucleotides based on their lengths (21-mers) and uniqueness when analyzed against the chicken genome. Although micrograms of DNA could be amplified from nanogram quantities of starting material, it resulted in insufficient enrichment of GaHV-2 over host DNA as measured by qPCR. There are two likely explanations for this failure. First of all, the optimal extension temperature for Phi29 DNA polymerase is 30 °C, a temperature too low for stringent annealing of GaHV-2-specific primers. Secondly, because of the large size of the chicken genome (approx. 3 gigabases), GaHV-2 oligonucleotides could not be designed without containing stretches of 13–14 nucleotides with near perfect homology to chicken sequences. At the low extension temperature these oligonucleotides would prime equally across both the chicken and GaHV-2 genomes.

3.2. Exonuclease treatment prior to Phi29 DNA polymerase-directed amplification

Lacking the ability to amplify viral DNA preferentially over host DNA, it seemed logical to attempt the removal of cellular DNA prior to Phi29 amplification. Although this could be accomplished by excision of GaHV-2 DNA from gels using pulsed field electrophoresis, enough starting material would have to be present for visualization by ethidium bromide staining (Davidson and Borenshtain, 2003; Isfort et al., 1990). It was reasoned that exonucleases could be used to degrade linear and nicked cellular DNA without affecting the covalently closed circular (CCC) replicative form of GaHV-2. Such exonucleases are commercially available and used to degrade contaminating chromosomal DNA and nicked DNA during the purification of bacterial artificial chromosome (BAC) DNA (ATP-dependent exonuclease component of the large construct kit, Qiagen, Valencia, CA) (Azad et al., 1992; Poulsen, 2004). Time course and dilution experiments were designed to determine the amount of exonuclease needed to degrade purified chicken chromosomal DNA to completion. One unit was defined as the amount of the proprietary Qiagen ATP-dependent exonuclease (28 ng) required to degrade 1 μg of chicken DNA at 37 °C for 1 h as determined by spectrophotometric analysis (260 nm) and stained agarose gel visualization of solubilized ammonium acetate precipitates. These conditions were used in reconstitution experiments in which cellular DNA was spiked with varying amounts (500 ng to 500 fg) of covalently closed circular GaHV-2 BAC DNA. This spiking DNA was isolated using the large construct kit (Qiagen) which includes an ATP-dependent exonuclease step and therefore is covalently closed circular DNA. Using Phi 29 DNA polymerase and either GaHV-2-specific primers or random primers, it was determined that at least 12 pg circular GaHV-2 DNA/μg cellular DNA was needed for the successful amplification of GaHV-2 as

measured by quantitative PCR (qPCR) with GaHV-2-specific primers and probe. Therefore, if success was to be achieved with whole infected cell DNA preps, at least 6.1×10^4 copies of GaHV-2 had to be present per microgram of genomic DNA. To test this, 2.0 μg of whole cell DNA isolated from GaHV-2 infected cells was subjected to exonuclease degradation using a large excess of ATP-dependent exonuclease (17.8 U) and amplified using Phi29 DNA polymerase with random hexamers. Prior research had indicated that random hexamers on a mole-to mole basis generated more DNA than MDV-specific oligonucleotides. After 48 h of amplification, 6.5 μg of DNA were generated as measured spectrophotometrically (OD_{260} nm). Although this amount seemed encouraging, the specificity of the exonuclease treated-Phi29 amplified products had to be confirmed using GaHV-2-specific qPCR. As a control to determine the copy number in the starting material, 2.0 μg of whole cell GaHV-2 infected DNA was only treated with the exonuclease and not amplified. Quantitative PCR indicated that 3.4×10^3 genomes (C_t value 30.21) survived the exonuclease treatment. However, only 6.5×10^7 genomes (total) were calculated after Phi29 amplification (C_t value of 20.64/10 ng of DNA quantified). This amounts to 1.0×10^4 genomes/ng of DNA, two logs lower than the theoretical (2.5×10^6 genomes/ng) based on 100% MDV. Therefore, something, in addition to the GaHV-2 DNA, was being amplified. Since qPCR indicated that only 0.4% of total DNA was GaHV-2, the origin of the amplified DNA was determined by sequencing shotgun-cloned random fragments of the amplified products. Of 79 clones sequenced, all were found to contain non-MDV (most likely chicken) DNA. BLAST analysis (Altschul et al., 1990) of the sequencing data against the GenBank nucleotide database showed a significant bias toward specific regions of the chicken genome. Of the 40 clones which were matched against annotated chicken sequences, 73% had homology to one of three regions (subtelomeric repeats, Synaptodin coding sequence, or LOC770715). It was therefore assumed that the chromosomal ends (and possibly other regions) of the chicken genome are resistant to exonuclease degradation, probably based on their structure or association with other macromolecules (e.g. RNA), and that the replicative forms of GaHV-2 do not exist as covalently closed circles, perhaps damaged during isolation, or are present in low copy numbers relative to nuclease-resistant host DNA.

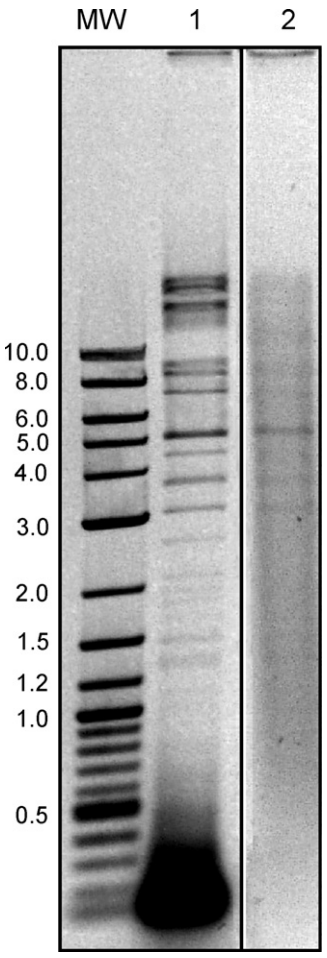

Fig. 1. Visualization of an ethidium bromide-stained gel (0.6% agarose) containing BamHI digested GaHV-2 DNA. MW: molecular weight marker, the 2-log DNA ladder (NEB); lane 1: GaHV-2 DNA isolated using the micrococcal nuclease procedure; and lane 2: GaHV-2 DNA isolated using hypotonic lysis follow by centrifugation through a 30% sucrose cushion (26).

3.3. Viral DNA isolation from cell-associated GaHV-2

Since neither of these approaches worked, a DNA isolation protocol that relied on a single round of *in vitro* propagation of GaHV-2 had to be developed. It had been reported that high quality viral DNA could be isolated from low-passage strains of a related virus, human cytomegalovirus (HCMV), using a hypotonic lysis/micrococcal nuclease digestion protocol (Sinzger et al., 1999). Because low-passage strains of HCMV are cell associated, similar to GaHV-2, it was postulated that this method might work for the purification of GaHV-2 DNA. Avian embryo fibroblasts (CEFs or DEFs) were infected with the CU-2 strain of GaHV-2 and after 4–6 days subjected to the hypotonic lysis/micrococcal nuclease protocol. The quality of the resulting DNA was assessed by digesting 1.0 μg of purified DNA with BamHI overnight followed by separation on a 0.6% agarose gel. Visualization of the DNA fragments by ethidium bromide staining (Fig. 1) indicated a dramatic reduction in background staining in the lane containing DNA isolated using the micrococcal nuclease procedure in comparison to the high background and barely visible viral bands in the lane containing DNA isolated without nuclease treatment (Spatz and Rue, 2008). Also evident was a large amount of nucleic acid of less than 1000 bp in the nuclease prep lane. When numerous RNases failed to remove the low-molecular weight nucleic acids, it was surmised to be degraded genomic DNA. In addition to rendering spectrophotometric measurements useless, this low-MW DNA posed a serious problem for the downstream application of pyrosequencing, which involves the production of small (~500 bp) randomly sheared GaHV-2 fragments for sequencing (Margulies et al., 2005). The contaminating species would be sequenced along with the GaHV-2 specific fragments.

3.4. Polyethylene glycol precipitation of high-molecular weight GaHV-2 DNA

Several methods (e.g. gel excision, agarose treatment, dialysis) were contemplated to remove the low-molecular weight degraded DNA present after micrococcal digestion, and it was determined that size-fractionation using an optimized polyethylene glycol (PEG) precipitation protocol could selectively precipitate large GaHV-2 genomes while retaining low-MW DNA fragments in the supernatant. Several publications have demonstrated the ability of PEG to precipitate nucleic acids differentially based on size (Lis, 1980; Lis and Schleif, 1975; Paithankar and Prasad, 1991; Schmitz and Riesner, 2006). It was shown that varying the concentration of PEG directly affected the minimum length of DNA that was precipitated. Therefore, the optimal concentration of PEG and centrifugation conditions needed to maximize precipitation of GaHV-2 genomic DNA while minimizing the amount of low MW DNA in the pellet had to be determined. To this end, gradient precipitations

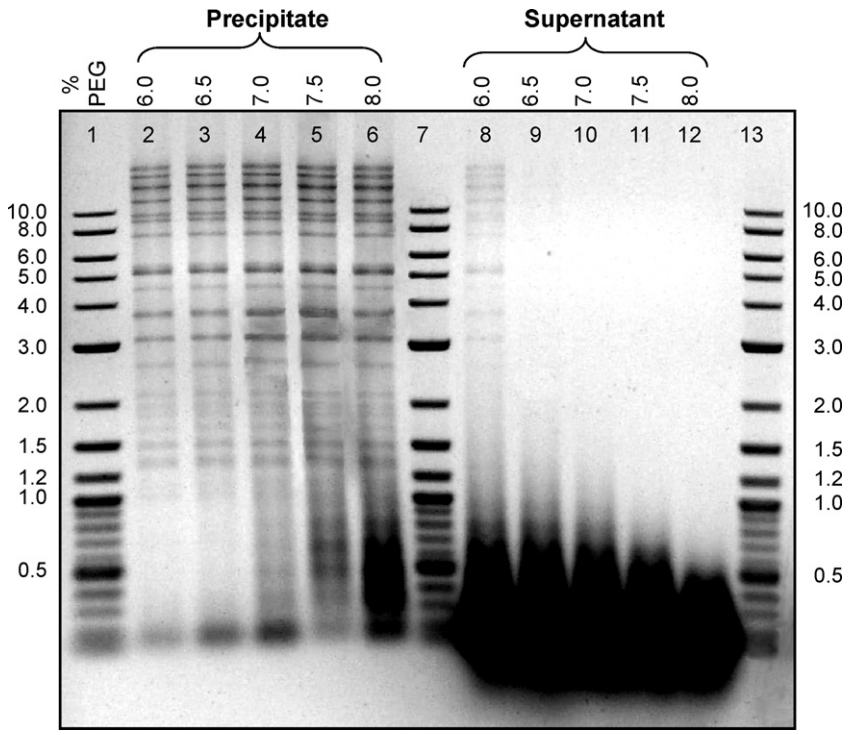

Fig. 2. Ethidium bromide-stained gel (0.6% agarose) containing BamHI digested GaHV-2 DNA recovered from the PEG pellet (lanes 2–5) and the matching supernatants (lanes 8–12). The percentages of PEG-8000 used in the precipitations are indicated at the top of the gel. Molecular weight markers (lanes 1, 7 and 13) are the 2-log DNA ladder (NEB).

were performed on a single nuclease-treated DNA prep using PEG-8000 concentrations ranging from 6.0 to 8.0% at 0.5% intervals. The low MW DNA present in the supernatants was precipitated with ammonium acetate and ethanol, and compared with the high MW DNA pellets using gel electrophoresis. Each DNA prep was digested with BamHI overnight and separated on a 0.6% agarose gel. Visual inspection of the ethidium bromide stained gel (Fig. 2) and quantitation at 260 nm of the undigested DNA indicated a significant reduction in the amount of contaminating DNA in the preps precipitated with final PEG-8000 concentrations ranging from 6 to 7%. A concentration of 6.5% PEG was determined to give an optimal balance of yield and purity. At lower concentrations, significant amounts of viral DNA fail to precipitate (lane 8 vs. lane 2), while at higher PEG concentrations contamination of the viral DNA pellet with low-MW host DNA increases greatly (lanes 5 and 6).

3.5. Pyrosequencing of GaHV-2 DNA

In order to determine the nucleotide sequences of six passages of the strain 648A, viral DNA was isolated using the optimized nuclease/PEG protocol and processed for 454 pyrosequencing. Depending on the passage, between 6 and 12 flasks (F75) infected

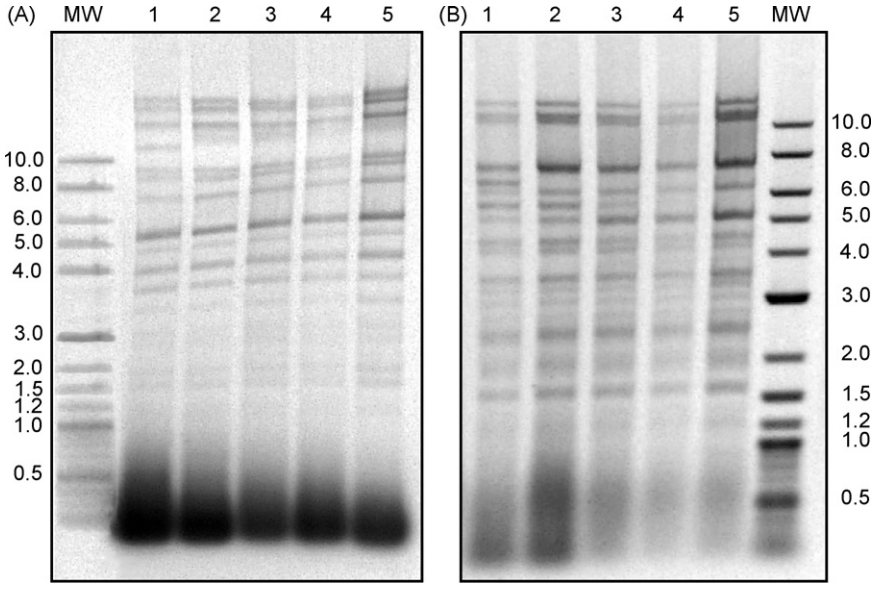

Fig. 3. Ethidium bromide-stained gels (0.6% agarose) containing restriction endonuclease digested DNA isolated using the micrococcal nuclease procedure prior to PEG 8000 precipitation (A) and after precipitation (B). DNA separated in gel (A) was digested with BamHI. DNA separated in gel (B) was digested with EcoRI. DNA in lane 1 is from passage 31; lane 2, 41; lane 3, 61; lane 4, 81 and lane 5, 101. Molecular weights (MW) are the 2-log DNA ladder (NEB).

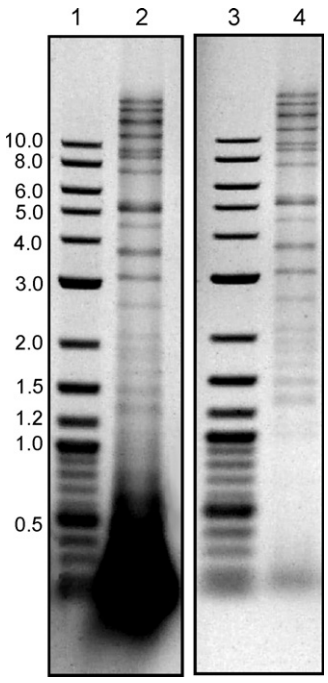

Fig. 4. Ethidium bromide-stained gels (0.6% agarose) containing BamHI digested DEF-passage p11 DNA purified using the micrococcal nuclease procedure (lane 2) and further purified using the PEG-8000 precipitation step (lane 4). Molecular weights (lanes 1 and 3) are the 2-log DNA ladder (NEB).

at an m.o.i. of 1.0, were needed to yield 5–10 μg of DNA. The quality of the DNA (p31–p101) was assessed both before PEG precipitation (Fig. 3A) and after (Fig. 3B) by separating restriction endonuclease-digested aliquots (500 ng) on 0.6% agarose gels. Passages 31–p101 generated large amounts of viral DNA when propagated on chick embryo fibroblast (CEF). This was not the case with p11. This passage closely resembles a field isolate and is poorly adapted to CEFs. In order to propagate this passage, 16 F75 flasks containing duck embryo fibroblasts were used to generate the 5.0 μg of DNA (Fig. 4; lane 4) needed for 454-pyrosequencing DNA.

Table 2 contains pyrosequencing data provided by 454 Life Sciences of the 6 GaHV-2 passages. The average read length was 179 bp with an average of 45,663 runs per GaHV-2 genome. The number of runs excludes those that were *Gallus gallus* specific (contaminating low MW DNA). The percentage cover varied over a wide range from 25 to 75-fold depending on the amounts of contaminating *Gallus gallus* DNA and the number of successful runs. This latter parameter is independent of the purity of the DNA and influenced by the chemistries involved in pyrosequencing. For example, p11 contained only 2.32% contaminating *Gallus gallus* DNA and only produced 42589 GaHV-2-specific runs for 44-fold coverage while p101 contained 20% *Gallus gallus* DNA with 67307 GaHV-2-specific runs for a 73-fold coverage. Generally DNA preps with low amounts of *Gallus gallus* DNA contamination yield higher fold coverage thus making contig assembly easier.

The percentage *Gallus gallus* DNA in Table 2 correlates well with the amount of low MW DNA on the agarose gels in Figs. 2 and 3. Any visible contamination in the 300–800 bp range results in a dramatic reduction in DNA purity, as can be seen with p41 (Fig. 3B lane 2). Very low MW residual contamination of around 100 bp in the p11 prep (Fig. 4; lane 4) does not appear to have a noticeable effect on 454 sequencing output, presumably because this DNA is lost during the fractionation (e.g. 500–800 bp) of sample DNA preps prior to the bead coupling step in the 454 pyrosequencing protocol.

The use of micrococcal nuclease digestion followed by PEG precipitation routinely yields microgram amounts of greater than 75% pure GaHV-2 DNA suitable for pyrosequencing. Since the ability to generate microgram quantities of viral DNA without further passage in cell culture (e.g. directly from infected tissues) was desired, PEG-precipitated DNA was tested for its suitability in Phi29 DNA amplification. Ten units of Phi29 DNA polymerase were used to amplify 300 ng of p11 DNA at 30 °C for 18 h using random hexamers. This reaction yielded approximately 9 μg of DNA, a 30-fold increase over the starting material. The amplified product was mechanically sheared to yield fragments averaging 700 bp in size and cloned in a pCR-BluntII TOPO vector (Invitrogen, Carlsbad, CA). Of nine clones that were subsequently sequenced, all nine contained GaHV-2 sequence, giving an indication of the purity of the PEG-precipitated DNA and its suitability for both direct pyrosequencing and further amplification using isothermal polymerases. These results suggest the possibility of isolating DNA from GaHV-2 infected organs (e.g. spleen or liver) for direct pyrosequencing using the micrococcal nuclease and PEG procedure followed by amplification using Phi29 DNA polymerase. Sequences at the termini of a DNA target are underrepresented in Phi29 amplification (Peters et al., 2006) which could be problematic. However, based on the genomic structure of GaHV-2, a class E herpesvirus, the termini (terminal repeat long and terminal repeat short regions) are inverted repeats of internal subgenomic regions (internal repeat long and internal repeat short, respectively) and therefore will not affect the fold coverage.

In conclusion, the isolation of viral DNA from GaHV-2 infected fibroblasts using the hypotonic lysis/micrococcal nuclease procedure originally described for a similarly cell-associated herpesvirus (low passage human cytomegalovirus) yielded high-molecular weight DNA suitable for restriction endonuclease profiling with minimal cellular DNA background. However, DNA purified this way was still contaminated with degraded DNA that could affect downstream applications (e.g. pyrosequencing). To remove this low-molecular weight DNA, polyethylene glycol was used to enrich for the high-molecular weight viral DNA. Using hypotonic lysis/micrococcal degradation in combination with the optimized

Table 2
Percentage coverage over the 117,500 bp GaHV-2 genomes using 454 pyrosequencing.

Sample	p11	p31	p41	p61	p81	p101
Total reads #	43,601	55,550	75,127	67,898	78,813	84,144
% MDV	97.68%	61.07%	37.79%	58.84%	78.44%	79.99%
MDV reads #	42,589	33,917	28,394	39,953	61,819	67,307
%*Gallus gallus*	2.32%	38.94%	62.21%	41.16%	21.56%	20.01%
Gallus gallus reads #	1,012	21,633	46,733	27,945	16,994	16,837
Ave read length	183	171.5	159	174	193.5	194
Amount of nucleotides	7,793,787	5,816,766	4,514,646	6,951,822	11,961,977	13,057,558
Fold coverage	43.9	32.8	25.4	39.2	67.4	73.6

454 Life Sciences sequencing results. *Gallus gallus* contamination is present in the six samples (p11, p31, p41, p61, p81 and p101) due to incomplete removal of the low-molecular weight DNA during 6.5% PEG precipitation. All the reads were mapped against *Gallus gallus* genome sequences with BLAST software (Altschul et al., 1990) and contaminating sequences were removed prior to contig assembly. Fold coverage was calculated as the number of GaHV-2 reads times the average read length divided by the average length (177,500 bp) of the GaHV-2 genome.

PEG precipitation, a 25–44-fold coverage using 454 Life Sciences pyrosequencing was obtained for genomes 177,500 nucleotides in length (based on 8 genomes/plate). It is postulated that these methods will be very useful for the isolation of viral DNA from cell cultures infected with other cell-associated viruses such as varicella-zoster virus, ovine herpesvirus 2, alcelaphine herpesvirus 1, and human herpesvirus 6, and possibly from tumors containing Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) DNA or Epstein-Barr viral DNA. When processed and sequenced using second and third generation sequencers, new characteristics of the viral population may begin to emerge and shed light on the possible quasi-species nature of DNA herpesviruses.

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